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# Synthesis and toxicological evaluation of newly synthesized 7,8-disubstituted theophylline derivatives

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**ABSTRACT**

*Four new 7,8-disubstituted theophylline derivartives were synthesized. Their purity was elucidated with melting points and TLC characteristics. The structure of the obtained compounds was proven by IR, $^{1}H$ NMR and $^{13}C$ NMR spectral data. The cytotoxicity, the in vitro effects on sub-cellular and cellular level and the antiproliferative activity of the synthesized compounds was evaluated. On isolated rat microsomes only **7d** showed statistically significant toxic effect. It increased the production of malondialdehyde by 52 % compared to the control. On isolated rat hepatocytes **7b** was outlined as the least toxic structure, while **7d** expressed highest toxicity, when compared to theophylline. Within the series only **7a** proved to be the most active antiproliferative agent, albeit at high micromolar concentrations.*

**Key words:** theophylline derivatives, isolated rat microsomes, isolated rat hepatocytes, cytotoxicity, antiproliferative activity.

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## INTRODUCTION

Natural products are an attractive source of varied structures that exhibit potent biological activities, and desirable pharmacological profiles. Xanthines have been an important class of biologically active structures. Theophylline (1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione) and a number of its 7-substituted or 7,8-disubstituted derivatives exhibit multidirectional pharmacological properties [1-3]. Their activities result from the complex mechanism including antagonism at adenosine receptors and non-selective inhibition of the cyclic nucleotide phosphodiesterases [4].

Along with the isomeric dimethylxanthine paraxanthine, which does not occure naturally, theophylline and theobromine are primary metabolic products of caffeine [5,6]. Caffeine, theophylline, theobromine and paraxanthine administered to animals and humans distribute in all body fluids and cross all biological membranes. They do not accumulate in organs and tissues and are extensively metabolized by the liver [7].

For theophylline was proved also antioxidant activity. It has been shown that theophylline may reverse the clinical observations of steroid insensitivity in patients with COPD and asthmatics that are active smokers (a condition resulting inoxidative stress) via a distinctly separate mechanism [8]. Theophylline *in vitro* can restore the reduced histone deacetylase (HDAC) activity that is induced by oxidative stress (i.e. in smokers), returning steroid responsiveness toward normal [9]. Furthermore, theophylline has been shown to directly activate HDAC2 as it is a

direct inhibitor of oxidant-activated phosphoinositide-3-kinase-delta, which is involved in inactivation of HDAC2 [10].

However, 8-thiosubstituted xanthines and the structures derived from them have not been well studied. There are 8-thio substituted derivatives of methylxanthines that possess radioprotector [11], antibacterial [12**]**, antidiabetic (as DPP-IV inhibitors) [13], and typical of methylxanthines bronchodilator activity [12**]**. Some 8-mercaptoxanthines have been used as intermediates for new purine-based heterocyclic ring systems, such as purinobenzothiazines and pyridothiazonopurines [14] which have shown antitumor activity.

The aim of our study is to investigate the toxicity of new 7,8-disubstituted theophylline derivatives in isolated rat microsomes and hepatocytes, and to compared with this of theophylline. Their possible antiproliferative activity in a pannel of tumor cell lines: HL-60, BV-173 and K-562 were also evaluated.

## MATERIALS AND METHODS

### Apparatuses and devices

The used chromatographic system for TLC control and purity elucidation is based on an alimunium sheets Silica gel F254 (Merck, Darmstadt, Germany), using the following mobile phases: *Phase 1:* 25% $NH_4OH$/Acetone/$CHCl_3$/$CH_3CH_2OH$ (1/3/3/4) and *Phase 2:* $H_2O$/n-butanol/$CH_3COOH$ (5/4/1), with detection at UV 254 nm. Yields were calculated for purified products. The IR spectra 400 – 4000$cm^{-1}$ were recorded on a Nicolet iS10 FT-IR Spectrometerin KBr pellets. $^1H$ and $^{13}C$-NMR spectra were registered on Bruker Spectrospin WM250 spectrometer (Faenlanden, Switzerland)at 250 and 75 MHz respectively, as δ (ppm) relative to TMS as internal standardand the coupling constants (*J*) are expressed inHertz (Hz). All NH protons were $D_2O$ exchangeable. Elemental analyses were performed by the microanalytical laboratory of Faculty of Pharmacy (Medical University-Sofia) on Euro EA 3000-Single, EUROVECTOR SpA analyser. All names were generated by using structure –to – name algorithm of ChemBioDraw Ultra software, Version 11.0, CambridgeSoft.The starting materials were of commercially available research – grade chemicals. (Merck, Darmstadt, Germany). Commercial aliquat 336 was used as catalysts.

### Synthesis of 7-benzyl-8-bromotheophylline (3)

In a round bottom flask equipped with mechanical stirrer and a reflux condenser 0.04 mol of 8-bromotheophylline and 0.06 mol of finely powdered KOH are suspended in 50 ml benzyl chloride. A half milliliter of the catalyst aliquot 336 is added and the temperature is raised under a glycerin bath up to 150$^o$C for one hour. The reaction was controlled by TLC and stopped after exhaustion of the starting compounds. After cooling a volumetric precipitate of 7-benzyl-8-bromotheophylline is formed. The obtained crystals were filtered and washed with petroleum ether. After drying out the crystals are poured in 50 ml of water and filtered again. The filtrate is evaporated and the unreacted benzyl chloride is regenerated. The yield after purification is 98%, m.p. – 175-177$^o$C. IR $\nu_{max}$ ($cm^{-1}$): 3121, 3062, 1718, 1668, 1566, 1485, 1445, 743.$^1H$ NMR (250 MHz, DMSO-$d_6$): 3.48 (s, 3H,N3-$\underline{CH_3}$), 3.31 (s, 3H, N1-$\underline{CH_3}$), 5.27 (s, 2H, N7-$\underline{CH_2}$), 6.97-7.17 (m, 5H, Ar-$\underline{H}$); $^{13}C$ NMR (75 MHz, DMSO-$d_6$): 154.7 (C6xanth CO); 151.4 ($C2_{xanth}$ CO); 148.8 ($C5_{xanth}$); 121.0 ($C8_{xanth}$); 136.7, 128.9, 128.6, 127.9. (6x C aromatic rings); 107.1 ($C4_{xanth}$); 53.7 ($N7_{xant}$ –$CH_2$), 27.9 ($N1_{xanth}$ $CH_3$), 29.8 ($N3_{xanth}$ $CH_3$). For $C14H_{13}BrN_4O_3$ (Mm = 349.19) calculated: C 48.16, H 3.75, Br 22.88, N 16.04; found: C 48.08, H 3.55, Br 22.58, N 16.00.

### Synthesis of 7-benzyltheophylline-8-thioacetic acid (4)

The reaction was carried out in reactor of 500 ml capacity which was equipped with mechanical stirrer (speed was maintained at 700 rpm) and a reflux condenser was placed on top of the reactor in order to prevent the escape of volatile compounds. The reaction mixture is water: ethanol = 40:60. Sodium hydroxide (0.06 mol) was preliminary solved in the water phase. Afterwards 0.03 mol of thioacetic acid were added. The ethanol and 7-benzyl-8-bromotheophylline (0.029 mol) were added and the reaction mixture was heated under reflux for 5 hours. The reaction mixture is filtered when hot and acidified with previously prepared solution of 1 part acetic acid and 4 parts water, to pH about 2. A white precipitate is obtained, and after filtration dissolved in 5% sodium hydrogen carbonate. The precipitate of the unreacted 7-benzyl-8-bromotheophylline is formed. The mixture is filtered and the filtrate is acidified with HCl to pH about 3. The separated precipitate is filtered under vacuum and dried. Yield – 85%, m.p. – 190 – 192°C. IR $\nu_{max}$ ($cm^{-1}$): 3441, 3166, 3001, 1737, 1710, 1664, 1554, 1486, 1438, 684. $^1H$ NMR (250 MHz, DMSO-$d_6$): 3.91 (s, 3H,N3-$\underline{CH_3}$), 3.44 (s, 3H, N1-$\underline{CH_3}$), 3.81 (s, 2H, S-$\underline{CH_2}$), 5.27 (s, 2H, N7-$\underline{CH_2}$), 6.97-7.23 (m, 5H, Ar-$\underline{H}$); $^{13}C$ NMR (75 MHz, DMSO-$d_6$): 171.4 (side chain –CO–),154.7 ($C6_{xanth}$ CO); 151.4 ($C2_{xanth}$ CO); 148.8 ($C5_{xanth}$); 147.0 ($C8_{xanth}$); 136.7, 128.9, 128.6, 127.9. (6x C aromatic rings); 107.1 ($C4_{xanth}$); 53.7 ($N7_{xant}$ –$CH_2$), 32.5 (side chain –S–$CH_2$–), 27.9 ($N1_{xanth}$ $CH_3$),29.8 ($N3_{xanth}$ $CH_3$). For $C_{16}H_{16}N_4O_4S$ (Mm = 360.39) calculated: C 53.32, H 4.47, N 15.55, S 8.90; found: C 53.45, H 4.44, N 15.21, S 8.85.

**Synthesis of methyl ester of 1-benzyltheotheophylline-8-thioacetic acid (5)**

Esterification of 7-benzyltheophylline-8-thioacetic acid was performed according to the classical esterification by interaction of the corresponding acid and alcohol in presence of sulfuric acid. The reaction was carried out in round bottom flask in excess of anhydrous methanol (300 ml). The flask is placed in a cooling bath and 30 ml of $H_2SO_4$ are added in aliquots. After its full addition 17g of 7-benzyltheophylline-8-thioaceticacid were added and the mixture was boiled under reflux for 4 hours. The process was monitored by TLC until exhaustion of the initial thioacetic acid. After the end of the reaction 2/3 of the solvent was removed under reduced pressure. and a triple amount of water was added to the the concentrated solution. The mixture was kept for 24 hours at 12$^o$C until formation of a volumetric precipitate, which was filtered and purified by re-crystallization from ethanol. Yield – 95%, m.p. – 159 – 161°C.IR $\nu_{max}$ (cm$^{-1}$): 3166, 3001, 1715, 1710, 1664, 1554, 1486, 1438, 1220, 688.$^1$H NMR (250 MHz, DMSO-*d*$_6$):3.82 (s, 3H,N3-$\underline{CH_3}$), 3.35 (s, 3H, N1-$\underline{CH_3}$), 3.54 (s, 2H, S-$\underline{CH_2}$), 3.68 (s, 3H, O-$\underline{CH_3}$), 5.27 (s, 2H, N7-$\underline{CH_2}$),6.97-7.23 (m, 5H, Ar-$\underline{H}$); $^{13}$C NMR(75 MHz, DMSO-*d*$_6$): 170.4 (side chain –CO–),154.7 (C6$_{xanth}$ CO); 151.4 (C2$_{xanth}$ CO); 148.8 (C5$_{xanth}$); 147.0 (C8$_{xanth}$); 136.7, 128.9, 128.6, 127.9 (6x C aromatic rings); 107.1 (C4$_{xanth}$); 53.7 (N7$_{xant}$ –CH$_2$), 52.3 (O–CH$_3$), 32.5 (side chain –S–CH$_2$–), 27.9 (N1$_{xanth}$ CH$_3$),29.8 (N3$_{xanth}$ CH$_3$). For $C_{17}H_{18}N_4O_4S$ (Mm = 374.41) calculated: C 54.54, H 4.85, N 14.96, S 8.56; found: C 54.45, H 4.45, N 14.86, S 8.45.

**General procedure for the synthesis of amides of 7-benzyltheophylline-8-thioacetic acid (7a-d)**

In a Kjeldahl flask connected to air condenser 0.005 mol of methyl ester of 7-benzyltheophylline-8-thioacetic acid (**5**) and 0.01mol of the corresponding amine (**6a-d**) were mixed and placed in glycerin bath at 115$^o$ – 130$^o$C respectively.The process was monitored by TLC until exhaustion of the initial amount of **5**. The obtained crystals were purified accordingly by re-crystallizationfrom ethanol and/or ether.

*2-(7-benzyl-1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)-N-phenethyl-acetamide* (**7a**): Reaction time: 60 min, Yield: 83%, M.p. 168-170°C, IR $\nu_{max}$ (cm$^{-1}$): 3293, 1716, 1690, 1651, 1644,1557, 1532, 1496, 1445, 696. $^1$H NMR (250 MHz, DMSO-*d*$_6$): 2.82 (t, 2H,*J*=6.5 Hz, Ar-$\underline{CH_2}$), 3.27 (t, 2H,*J*=6.5 Hz, N-$\underline{CH_2}$),3.39 (s, 3H,N3-$\underline{CH_3}$), 3.31 (s, 3H, N1-$\underline{CH_3}$), 3.68 (s, 2H, S-$\underline{CH_2}$), 5.27 (s, 2H, N7-$\underline{CH_2}$),6.97-7.31 (m, 10H, Ar-$\underline{H}$); $^{13}$C NMR(75 MHz, DMSO-*d*$_6$): 163.9 (side chain –CO–),154.7 (C6$_{xanth}$ CO); 151.4 (C2$_{xanth}$ CO); 147.0 (C8$_{xanth}$); 139.0, 136.7, 128.9, 128.7, 128.6, 128.5, 127.9. (12 x C aromatic rings); 148.8 (C5$_{xanth}$); 107.1 (C4$_{xanth}$); 53.7 (N7$_{xant}$ –CH$_2$), 41.4 (–CH$_2$–NH side chain), 35.7 (side chain –CH$_2$–), 32.1 (side chain –S–CH$_2$–), 27.9 (N1$_{xanth}$ CH$_3$),29.0 (N3$_{xanth}$ CH$_3$). For $C_{24}H_{25}N_5O_3S$ (Mm = 463.55) calculated: C 62.19, H 5.44, N 15.11, S 6.92; found: C 62.16, H 5.52, N 15.01, S 6.85.

*2-(7-benzyl-1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)-N-(3,4-dimethoxy-phenethyl)acetamide* (**7b**): Reaction time: 60 min, Yield: 70%, M.p. 158-159°C, IR $\nu_{max}$ (cm$^{-1}$): 3296, 2830, 1725, 1692, 1660, 1644,1557, 1516, 1494, 1454, 1230, 696. $^1$H NMR (250 MHz, DMSO-*d6*): 2.61 (t, 2H, *J*=6.5 Hz, Ar-$\underline{CH_2}$), 3.82 (t, 2H, *J*=6.5 Hz, N-$\underline{CH_2}$), 3.48 (s, 3H, N3-$\underline{CH_3}$), 3.73 (s, 3H, O-$\underline{CH_3}$), 3.80 (s, 3H, O-$\underline{CH_3}$), 3.32 (s, 3H, N1-$\underline{CH_3}$), 3.68 (s, 2H, S-$\underline{CH_2}$), 5.27 (s, 2H, N7-$\underline{CH_2}$), 6.52-7.23 (m, 8H, Ar-$\underline{H}$); $^{13}$C NMR(75 MHz, DMSO-*d6*): 163.9 (side chain –CO–),154.7 (C6$_{xanth}$ CO); 151.4 (C2$_{xanth}$ CO); 147.0 (C8$_{xanth}$); 149.2, 148.1, 136.7, 130.9, 128.9, 128.6, 127.9, 120.8, 111,62(12 x C aromatic rings); 148.8 (C5$_{xanth}$); 107.1 (C4$_{xanth}$); 53.7 (N7$_{xant}$ –CH$_2$), 41.4 (–CH$_2$–NH side chain), 34.7 (side chain –CH$_2$–), 32.1 (side chain –S–CH$_2$–), 27.9 (N1$_{xanth}$ CH$_3$),29.0 (N3$_{xanth}$ CH$_3$). For $C_{26}H_{29}N_5O_5S$ (Mm = 523.61) calculated: C 59.64, H 5.58, N 13.38, S 6.12; found: C 59.69, H 5.55, N 13.31, S 6.05.

*2-(7-benzyl-1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)-N-(4-hydroxy-phenethyl)acetamide* (**7c**): Reactiontime: 45min, Yield: 80%, M.p. 239-241°C, IR $\nu_{max}$ (cm$^{-1}$): 3282, 2931, 1716, 1695, 1660, 1648, 15998, 1506, 1497, 1448, 1232, 698. $^1$H NMR (250 MHz, DMSO-*d*$_6$): 2.72 (t, 2H, *J*=6.8 Hz, Ar-$\underline{CH_2}$), 3.31 (t, 2H, *J*=6.8 Hz, N-$\underline{CH_2}$), 3.48 (s, 3H, N3-$\underline{CH_3}$), 3.42 (s, 3H, N1-$\underline{CH_3}$), 3.68 (s, 2H, S-$\underline{CH_2}$), 5.27 (s, 2H, N7-$\underline{CH_2}$), 6.7-7.23 (m, 9H, Ar-H); $^{13}$C NMR(75 MHz, DMSO-*d*$_6$): 163.9 (side chain –CO–),154.7 (C6$_{xanth}$ CO); 151.4 (C2$_{xanth}$ CO); 147.1(C8$_{xanth}$); 157.8, 136.7, 130.7, 132.3, 128.9, 128.6, 127.9, (12 x C aromatic rings); 148.8 (C5$_{xanth}$); 107.1 (C4$_{xanth}$); 53.7 (N7$_{xant}$ –CH$_2$), 41.4 (–CH$_2$–NH side chain), 35.75 (side chain –CH$_2$–), 32.1 (side chain –S–CH$_2$–), 27.9 (N1$_{xanth}$ CH$_3$), 29.0 (N3$_{xanth}$ CH$_3$). For $C_{24}H_{25}N_5O_4S$ (Mm = 479.55) calculated: C 60.11, H 5.25, N 14.60, S 6.69; found: C 59.69, H 5.55, N 14.38, S 6.55.

*2-(7-benzyl-1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylthio)-N-(1-phenyl-propan-2-yl)acetamide* (**7d**): Reactiontime: 45 min, Yield: 75%, M.p. 172-174°C, IR $\nu_{max}$ (cm$^{-1}$): 3278, 1721, 1691, 1651, 1604, 1558, 1533, 1497, 1448, 700. $^1$H NMR (250 MHz, DMSO-*d*$_6$): 1.43 (d, 3H, *J*=6.7 Hz,N-CH-$\underline{CH_3}$), 2.72 (t, 2H, *J*=6.3 Hz, Ar-$\underline{CH_2}$), 4.31 (q, 1H, *J*=6.7 Hz,N-$\underline{CH}$), 3.46 (s, 3H, N3-$\underline{CH_3}$), 3.31 (s, 3H, N1-$\underline{CH_3}$), 3.68 (s, 2H, S-$\underline{CH_2}$), 5.27 (s, 2H, N7-$\underline{CH_2}$), 6.96-7.28 (m, 10H, Ar-H); $^{13}$C NMR (75 MHz, DMSO-*d*$_6$): 168.5 (side chain –CO–), 154.0 (C6$_{xanth}$ CO); 151.3 (C2$_{xanth}$ CO); 147.0 (C8$_{xanth}$); 137.3, 136.7, 129.1, 128.9, 128.7, 128.6, 127.9 (12 x C aromatic rings); 148.8 (C5$_{xanth}$); 107.9 (C4$_{xanth}$); 51.4 (–CH–NH side chain), 53.7 (N7$_{xanth}$ –CH$_2$), 38.8 (side chain –CH$_2$–), 32.1 (side chain

–S–$CH_2$–), 29.8 ($N3_{xanth}$ $CH_3$), 27.9 ($N1_{xanth}$ $CH_3$), 19.5 (side chain –$CH_3$). For $C_{25}H_{27}N_5O_3S$ (Mm = 477.58) calculated: C 62.87, H 5.70, N 14.66, S 6.71; found: C 62.71, H 5.61, N 14.35, S 6.65.

***Chemicals***

In our experiments, pentobarbital sodium (Sanofi, France), HEPES (SigmaAldrich, Germany), NaCl (Merck, Germany), KCl (Merck), D-glucose (Merck), $NaHCO_3$ (Merck), $KH_2PO_4$ (ScharlauChemieSA, Spain), $K_2HPO_4$ (ScharlauChemieSA, Spain), Glycerol (ScharlauChemieSA, Spain), $CaCl_2.2H_2O$ (Merck), $MgSO_4.7H_2O$ (FlukaAG, Germany), collagenase from *Clostridium histolyticum* type IV (Sigma Aldrich), albumin, bovine serum fractionV, minimum 98 % (Sigma Aldrich), EGTA (Sigma Aldrich), 2-thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol; TBA) (Sigma Aldrich), trichloroacetic acid (TCA) (Valerus, Bulgaria), 2,2'-dinitro-5,5'-dithiodibenzoicacid (DTNB) (Merck), lactatedehydrogenase (LDH) kit (Randox, UK) were used.

***Animals***

The male Wistar rats (body weight 200–250 g) were housed in plexiglass cages (3 per cage) in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature 20°C ± 2°C and humidity 72 % ± 4 %) with free access to water and standard pelleted rat food 53-3, produced according ISO 9001:2008.

Animals were purchased from the National Breeding Center, Sofia, Bulgaria. At least 7 days of acclimatization was allowed before the commencement of the study. The health was monitored regularly by a veterinary physician. The vivarium (certificate of registration of farm № 0072/01.08.2007) was inspected by the Bulgarian Drug Agency in order to check the husbandry conditions (№ A-11-1081/03.11.2011). All performed procedures were approved by the Institutional Animal Care Committee and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) [15] were strictly followed throughout the experiment.

***Isolation and incubation of microsomes***

The liver was perfused by 15 % cold KCl and after that was minced and homogenate with 0.1M phosphate buffer pH 7.5. The homogenate was centrifuged at 9000 rpm (4°C) for 30 min. The supernatant (S-9 fraction) was divided in centrifuged tubes and ultracentrifuged at 106 000 rpm (4°C), in a Beckman L8-M centrifuge with a 70Ti rotor, for 1 hour. The pellet was covered with 1 ml phosphate/glycerol buffer (20 % glycerol and 80 % 0.1 M phosphate buffer pH 7.5) and freezed [16].

*Protein content* was measured according to the method of Lowry et al. [17].

***Isolation and incubation of hepatocytes***

Rats were anesthetized with sodium pentobarbital (0.2 ml/100 g). *In situ* liver perfusion and cell isolation were performed as described by Fau et al. [19] with modifications [20].

After portal catheterization, the liver was perfused with HEPES buffer (pH = 7.85) + 0.6 mM EDTA (pH = 7.85), followed by HEPES buffer (pH = 7.85) without any addition and finally HEPES buffer, containing collagenase type IV (50 mg/200 ml) and 7 mM $CaCl_2$ (pH = 7.85). The liver was excised, minced into small pieces and hepatocytes were dispersed in Krebs-Ringer-bicarbonate (KRB) buffer (pH = 7.35) + 1% bovine serum albumin.

Cells were counted and the viability was assessed by Trypan blue exclusion (0.05 %) [19]. Initial viability averaged 86 %.

Cells were diluted with KRB, to make a suspension of about 3 x $10^6$ hepatocytes/ml. Incubations were carried out in flasks, containing 3 ml of the cell suspension (i.e. 9 x $10^6$ hepatocytes) and were performed in a 5 % $CO_2$ + 95 % $O_2$ atmosphere [19].

***Biochemical parameters***

*Malondialdehyde (MDA) formation assay in isolated rat microsomes*

After 30 min incubation of the microsomes (1 mg/ml protein) with the compounds, the reaction was stopped with 1 ml 25 % trichloracetic acid and 1 ml 0.67 % 2-thiobarbituric acid. The mixture was heated at 100°C for 20 min and after that was centrifuged at 4 000 rpm for 10 min. The production of malondialdehyde was measured spectrophotometrically at 535 nm [18].

*MDA assay in isolated hepatocytes*

Hepatocyte suspension (1 ml) was taken and added to 0.67 ml of 20 % (w/v) TCA. After centrifugation, 1 ml of the supernatant was added to 0.33 ml of 0.67% (w/v) 2-thiobarbituric acid (TBA) and heated at 100°C for 30 min. The

absorbance was measured at 535 nm, and the amount of TBA-reactants was calculated using a molar extinction coefficient of MDA 1.56 x $10^5$ $M^{-1}cm^{-1}$ [19].

*Lactate dehydrogenase release*
Lactate dehydrogenase (LDH) release in isolated rat hepatocytes was measured as described by Bergmeyer et al. [21], by using LDH kit.

*Glutathione assay*
At the end of the incubation, isolated rat hepatocytes were recovered by centrifugation at 4°C, and used to measure intracellular reduced glutathione (GSH), which was assessed by measuring non-protein sulfhydryls after precipitation of proteins with trichloroacetic acid (TCA), followed by measurement of thiols in the supernatant with DTNB. The absorbance was measured at 412 nm [19].

***Cell lines and culture conditions***
In this study the following human tumor cell lines were used: HL-60 (acute myelocyte leukemia), BV-173 and K-562 (chronic myeloid leukemias). They were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). The cells were grown in controlled environment – cell culture flasks at 37°C in an incubator 'BB 16-Function Line' Heraeus (Kendro, Hanau, Germany) with humidified atmosphere and 5% $CO_2$. Cells were kept in *log* phase by supplementation with fresh medium after removal of cell suspension aliquots, two or three times a week. The cell lines were maintained in 90% RPMI-1640 + 10% FBS.

*Cytotoxicity assessment (MTT-dye reduction assay).*
The cell viability was assessed using the standard MTT-dye reduction assay as described by Mosmann [22] with minor modifications [23]. The method is based on the reduction of the yellow tetrazolium salt MTT to a violet formazan product *via* the mitochondrial succinate dehydrogenase in viable cells. Exponentially growing cells were seeded in 96-well flat-bottomed microplates (100 µl/well) at a density of $1\times10^5$ cells per ml and after 24h incubation at 37°C they were exposed to various concentrations of the tested compounds for 72h. For each concentration a set of at least 8 wells were used. After the exposure period 10µl MTT solution (10mg/ml in PBS) aliquots were added to each well. Thereafter the microplates were incubated for 4h at 37°C and the MTT-formazan crystals formed were dissolved through addition of 100 µl/well 5% formic acid (in 2-propanol). The absorption was measured using a LabeximLMR-1 microplate reader at 580 nm. Cell survival fractions were calculated as percentage of the untreated control.

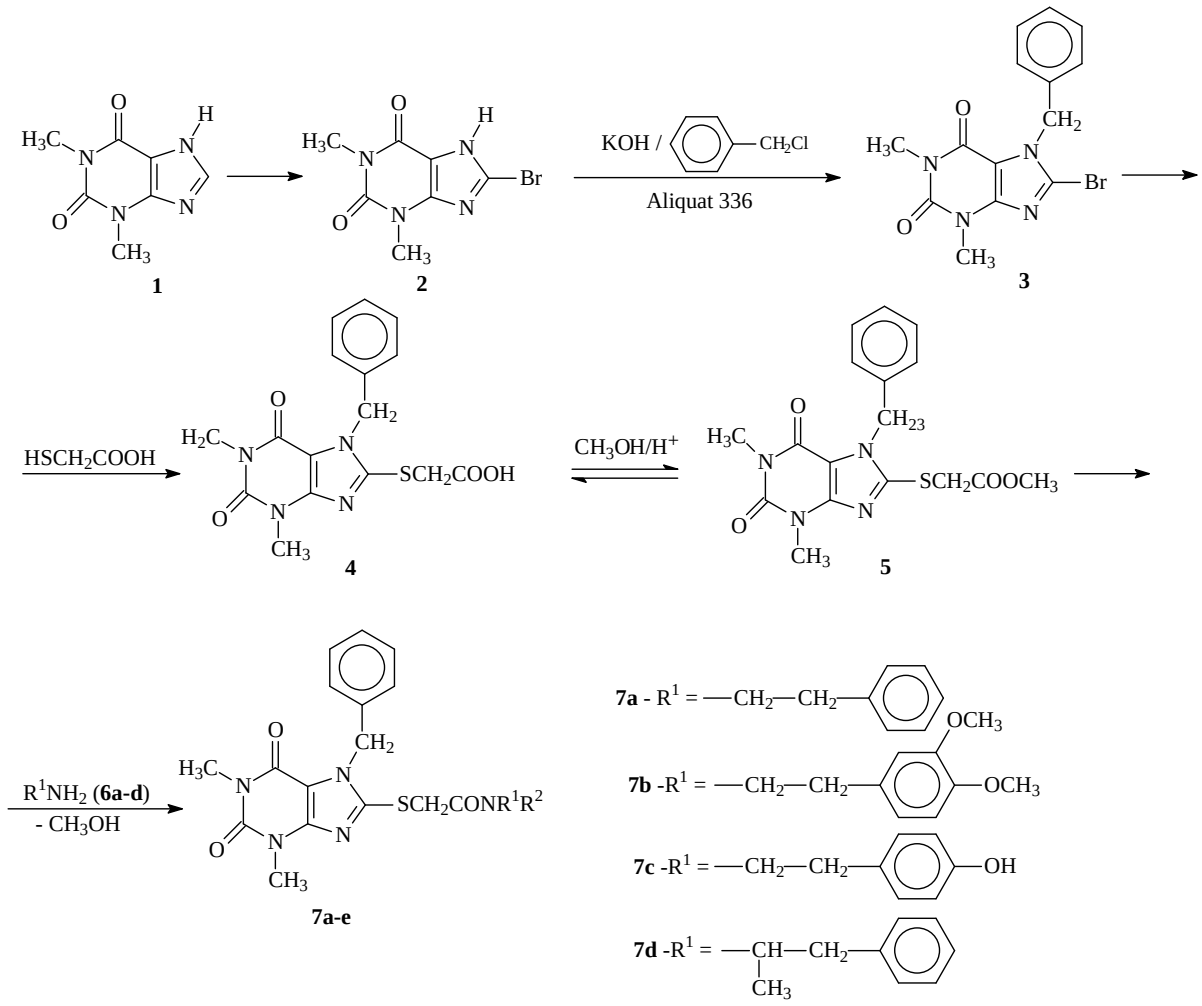
***Statistical analysis***
The MTT-bioassay raw data were normalized as survival fractions (%) relative to the untreated control (set as 100% viable), and the equieffective $IC_{50}$ values (concentrations causing half-maximal decrease of cell survival) were calculated using non-linear regression ('Curve fit'-GraphPad Prizm Software for PC). The statistical processing of MTT data included the paired Student's t-test with $P \leq 0.05$ set as significance level.

Statistical analysis was performed using statistical programme 'MEDCALC'. Results are expressed as mean ± SEM for 7 experiments. The significance of the data was assessed using the nonparametric Mann–Whitney test. Values of $P \leq 0.05$; $P \leq 0.01$ and $P \leq 0.001$ were considered statistically significant. Three parallel samples were used.

## RESULTS AND DISCUSSION

**Chemistry**
The synthesis of amide derivatives of 7-benzyltheophylline-8-thioacetic acid consists of brominating of theophylline (**1**) to obtain 8-bromotheophylline (**2**). The synthesis of 8-bromotheophylline (**2**) was carried out via oxidative bromination of theophylline (**1**) according to the method published elsewhere [24], based on application of HBr and $H_2O_2$ in glacial acetic acid. At these conditions the reaction due 4 hours and the corresponding yield of re-crystallized 8-bromotheophylline was 80 –85%. Further interaction of **2** with benzyl chloride resulted in synthesis of 1-benzyl-8-bromotheophylline (**3**). The synthesis was performed under phase transfer catalysis conditions, whereat aliquot 336 was used as phase transfer catalyst. The next stage of our synthetic sequence is formation of the corresponding 7-benzyltheophylline-8-thioacetic acid (**4**) by interaction of (**3**) with thioacetic acid.Based on previous experiments improving the reaction conditions [25] we led the reaction in water/ethanol media, in the presence of sodium hydroxide. At these conditions no side reaction was observed and the reaction time was shortened up to 1 and a half hour with yield of 81 %. The necessary for the aminolysis ester derivative (**5**) was obtained by esterification. The targeted structures (**7a-d**) were synthesized by classical aminolysisof the methyl ester of 7-benzyltheophylline-8-thioacetic acid with of the corresponding amine **6a-d**. The aminolysis of the compounds was performed in melt, with 2:1 correlation of the reactants for the selected amine. For all the synthesis the reaction

mixture is heated in glycerin bath until the methyl ester of 7-benzyltheophylline-8-thioacetic acid is melted, which forms melt with the applied amine, and this temperature is maintained until the end of the reaction. The reaction times are between 20 min to one hour, the temperature vary from 110 to 130°C and the yields are in the range of 61 to 81,2%. The total synthetic scheme is presented on **Scheme 1**. Using the above described methods four amide derivatives of 7-benzyltheophylline-8-thioacetic acid were synthesized. The reaction conditions and the corresponding reaction time and yield for each of the targeted products are given in **Table 1**.




**Scheme 1. General scheme of synthesis of amides of 7-benzyltheophylline-8-thioacetic acid**

**Table 1. Reaction conditions and the corresponding reaction times, yields and melting points for the synthesis of each of the targeted products**

| compound | temperature °C | reaction time min. | Yield % | Melting point °C |
|---|---|---|---|---|
| **7a** | 120° | 60 | 83 | 168-170 |
| **7b** | 130° | 60 | 70 | 158-159 |
| **7c** | 130° | 45 | 80 | 239-241 |
| **7d** | 130° | 45 | 75 | 172-174 |

As seen in **Table 1** the products **7a-d** are obtained with good yields and purity, whereas **7a** is obtained with highest yield. The obtained compounds are white crystalline powders, soluble in organic solvents (chloroform, benzene, dimethylformamide) and practically insoluble in water. The structure of the newly synthesized compounds was proven by elemental analysis, FT-IR, $^{1}$HNMR, $^{13}$CNMR spectroscopy.

The FT-IR spectra of the final N-substituted7-benzyltheophylline-8-thioacetamides **7a-d** exhibit intensive several characteristic bands in the region of 4000 – 400cm$^{-1}$. The two carbonyl groups from the xanthine fragment absorb at 1651 to 1691 cm$^{-1}$. The vibrational band recorded at 1716 – 1725 cm$^{-1}$ belongs to the carbonyl in the amide moiety in the side chain (amide I), while the band at 1595 – 1605 cm$^{-1}$ in the spectra of **7a-d** corresponds to stretching vibration N-H from the same residue (amide II). The absorbance in the region 3278– 3296 cm$^{-1}$corresponds to the absorption frequencies of NH – stretching vibrations (amide A). The streching vibration of the C=C bonds from the

xanthine ring is responsible for the band at 1555 $cm^{-1}$, whereas the stretching vibrations of aromatic ring appears at 1445 $cm^{-1}$ and 1497 $cm^{-1}$. The absorbance at 2830 $cm^{-1}$ and 1232 $cm^{-1}$ in the spectrum of **7b** is due to stretching vibrations of C–H and aryl–O bonds respectively in the two $OCH_3$ groups. The methylene groups, as well as $N–CH_3$ groups are found in the region 2780 – 2970 $cm^{-1}$. In the spectra of all compounds a strong absorption at 696 $cm^{-1}$was observed and it can be attributed to the characteristic stretching vibration of C-S bond at 8$^{th}$ position. According to literature data, this value is intrinsic to vibration of this bond in arylthioethers [26].

More detailed information about the structure of compounds **7a-d** was provided by the $^1$H– and $^{13}$C–NMR spectra. Thus, the strong singlets at 3.31÷3.44 and 3.39÷3.91 ppm in the spectrum of the studied compounds correspond to N-methyl protons at position 1 and 3. It is noteworthy that the chemical shift values of the protons at 3$^{rd}$ position vary more widely. In the compounds **7a-d** they are approximately at 3.39 to 3.41 ppm, while in **3**, **4** and **5**, the values tend towards the upper limit of the range, which can be explained by the influence of substituents on the 8$^{th}$ place in xanthine ring. The signal of the S-methylene group from 7-benzyltheophylline-8-thioglycolic acid at position 8 in **7a-d** appears at 3.68 ppm as broad singlet compared to the signals for the same protons in the compound **4** (3.81 ppm) and **5** (3.54 ppm). Here very clearly was shown the influence of arylalkylamide fragment in the side chain. The signals of methylene protons from the benzyl side chain at position 7 form strong singlet at 5.27 ppm. The signals of the aromatic protons from benzyl side chain at position 7 in the spectra of **3**, **4**, **5** and **7a-d** correspond to complicated multiplets between 6.52 and 7.23 ppm, but the integral curves correspond to the exact number of the protons. The protons of the two methoxy groups in **7b** appear as strong singlets at 3.73 ppm and 3.80 ppm. In the spectrum of **7d** there is a strong singlet at 1.43 ppm that corresponds to methyl protons in the side chain as well as quartet at 4.31 ppm assigned to NH–CH proton. The values of the chemical shift and integral curve confirm the proposed structure of **7d**.

The data obtained from $^{13}$C NMR spectra are fully consistent with proposed structures. The values of the chemical shifts of the protons registered by $^1$H NMR spectra and carbons registered by $^{13}$C NMR spectra were compared with simulated values [27-30]. We observed only small deviations of computed from experimental values, due to an impossibility to render an account of influence of the solvent. Regardless, the simulated $^1$H– and $^{13}$C NMR spectra are in good correlation with experimental ones.

The obtained amides were tested for hydrolytic stability. It was determined, that they are stable to hydrolysis under acidic or alkali conditions, close to physiological pH and temperatures about 40$^o$C. Under these conditions no hydrolysis is observed for a period of more than 20 hours. The stability teste were performed accroding to the procedure described in [31].

**Antiproliferative activity**

The compounds were tested for antiproliferative activity in a panel of human tumor cell lines. The chronic myeloid leukemia derived BV173 proved to be the most responsive tumor model to the effects of the tested agents, whereas K-562 and HL-60 were less sensitive. The results are presented in **Table 2**.

**Table 2.Cytotoxic effects of the tested compounds in a panel of human tumor cell lines, after 72 h continuous exposure (MTT-dye reduction assay)**

| Compound | $IC_{50}$ (μM) | | |
|---|---|---|---|
| | *HL-60* | *BV-173* | *K-562* |
| **Cisplatin** | 5.75 | 6.08 | 13.77 |
| **7a** | 374.1 | 43.4 | 260.1 |
| **7b** | > 800 | 99.8 | > 800 |
| **7c** | >800 | > 800 | >800 |
| **7d** | >800 | 103.6 | >800 |

From the obtained data is visible, that within the series **7a** proved to be the most active antiproiliferative agent, albeit at high micromolar concentrations. Among the other tested compounds **7b** and **7d** demonstrated only marginal activity in HL-60 and K-562, causing 50% inhibitory effects in BV-173 only; **7c** was the least active compound, as demonstrated on **Fig.1**.

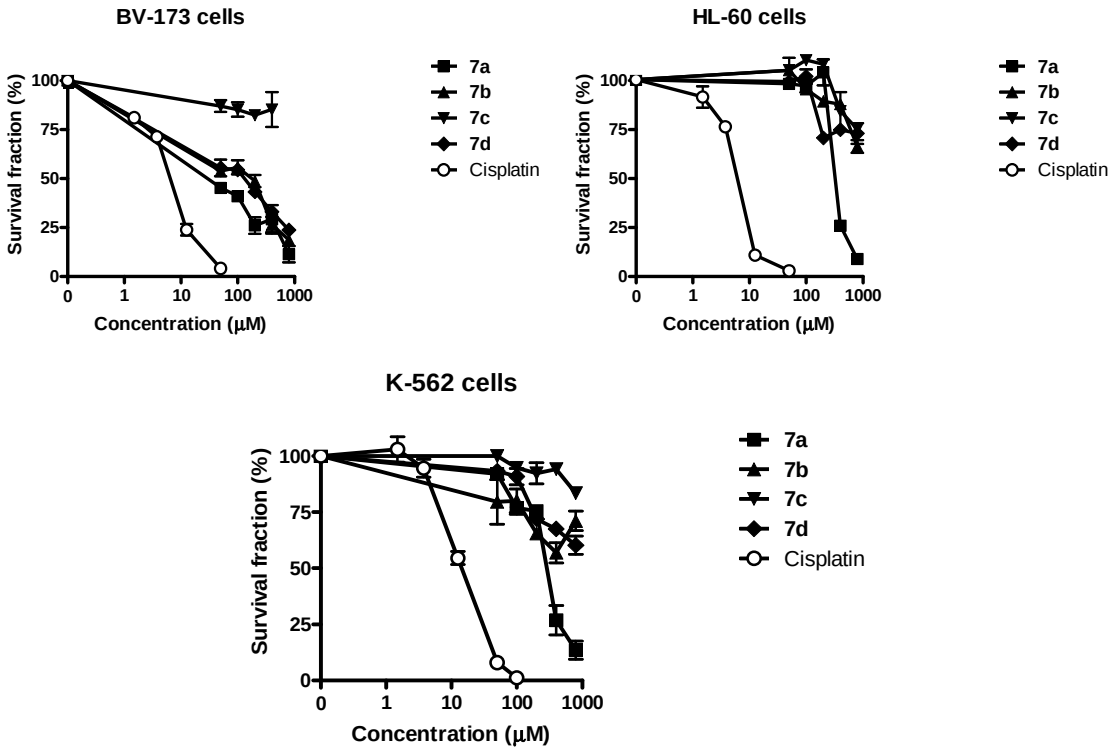



**Fig. 1. Antiprolipherative effect of tested compounds on human tumor cell lines after 72h incubation (MTT test)**

**Toxicological assessments in isolated rat liver hepatocytes and microsomes**

Liver monooxigenase system plays an important role in xanthine's metabolism and formation. It has been proven that isoform CYP2E1 (the ethanol-inducible form) and flavin-containing monooxygenase are catalysing the formation of theophylline and theobromine from caffeine [32-34]. In addition, Lelo et al. [5] found that the primary metabolic pathways of the natural xanthenes caffeine, theobromine and theophylline are N-demethylations and C(8)-oxidation. Thus, having in mind the addition of some S-containing substituents at 8$^{th}$ position of the theophylline core, it was of interest to determine whether the newly synthetized theophylline derivatives affect hepatocytes function by possible prooxidant activity and cytotoxic radical formation. The effects of derivatives **7a–d** (100μM) were evaluated on cell viability, LDH leakage and GSHdepletion in isolated rat hepatocytes.

***Lipid peroxidation study***

The antioxidant/prooxidant activity of the compounds were studied by evaluation of the levels of MDA production in isolated rat microsomes (**Table 3**).

We found that only **7d** shows a statistically significant toxic effect. Compound **7d** increased the production of MDA by 52 %, compared to the non-treated microsomes, used as a control. The initial theophylline and its new 7,8-disubstituted derivatives didn't induce lipid peroxidation in statistically significant manner.

**Table 3. Effects of theophylline (100 μM) and 7a-d derivatives (100 μM) on MDA production in isolated rat microsomes**

| Group | MDA (nmol/mg protein) | Effect (%) vs control |
|---|---|---|
| Control | 2,1± 0,1 | 100 |
| Theophylline | 2,1± 0,1 | ↓ 0 |
| **7a** | 2,2± 0,1 | ↑ 5 |
| **7b** | 1,98± 0,2 | ↓ 6 |
| **7c** | 1,97± 0,1 | ↓ 6 |
| **7d** | 3,2± 0,1 ** | ↑ 52 |

** *P< 0,01vscontrol*

MDA production was also determined in isolated rat hepatocytes as a main parameter for lipid peroxidation (**Table 4**). The initial theophylline increased MDA production statistically significant by 18 %, while **7b** didn't induce any lipid peroxidation. From the tested compounds only **7d** revealed strong pro-oxidant effect, increasing the MDA production by 38% ($P < 0,01$), compated to theophylline.

**Table 4. Effects of theophylline (100 µM) and 7a-d (100 µM) on MDA level in isolated rat hepatocytes**

| Group | MDA (nmol/mill cells) | Effect (%) vs control |
|---|---|---|
| Control | 0,04± 0,002 | 100 |
| Theophylline | 0,047± 0,003 * | ↑ 18 |
| **7a** | 0,046 ± 0,003 ** | ↑ 15 |
| **7b** | 0,043 ± 0,002 | ↑ 8 |
| **7c** | 0,050 ± 0,01 * | ↑ 25 |
| **7d** | 0,055± 0,01 ** | ↑ 38 |

** P < 0,05;**P < 0,01vs control*

***Cytotoxicity evaluation***

The cytotoxicity and hepatotoxicity of theophylline and its 7,8-disubstituted derivatives were evaluated by determination of cell viability, LDH leakage and GSH depletion in isolated rat hepatocytes. Theophylline (100 µM) decreased cell viability statistically significant by 29 %, compared to the control (non-treated hepatocytes); **7d** shows a similar cytotoxic effect to those of theophylline, reducing the cell viability by 25 %, while **7b** shows the lowest cytotoxic effect –15 % decrese vs control (**Table 5**).

**Table 5. Effects of theophylline (100 µM) and 7a-d (100 µM) on cell vibility in isolated rat hepatocytes**

| Group | Cell vibility (%) | Effect (%) vs control |
|---|---|---|
| Control | 79± 4,04 | 100 |
| Theophylline | 56 ± 9,2* | ↓ 29 |
| **7a** | 66±1,2* | ↓ 16 |
| **7b** | 67±1,2* | ↓ 15 |
| **7c** | 60±2,9* | ↓ 24 |
| **7d** | 59 ± 2,9* | ↓ 25 |

** P < 0,05vs control*

LDH leakage from the cells is another valuable parameter for cytotoxicity evaluation. It was observed that theophylline increased the LDH leakage statistically significant by 324 %, compared to the control. The derivative **7d** revealed toxic effect similar to those of theophylline increasing LDH leakage by 317 % *vs* control while **7b** didn't show any effect on this parameter (**Table 6**).

**Table 6. Effects of theophylline (100 µM) and 7a-d (100 µM) on LDH leakage in isolated rat hepatocytes**

| Group | LDH (µmol/min/$10^6$ cells) | Effect (%) vs control |
|---|---|---|
| Control | 0,120± 0,001 | 100 |
| Theophylline | 0,509 ± 0,03 ** | ↑ 324 |
| **7a** | 0,150± 0,03 | ↑ 25 |
| **7b** | 0,119± 0,001 | 0 |
| **7c** | 0,168± 0,01 * | ↑ 40 |
| **7d** | 0,500 ± 0,02 ** | ↑ 317 |

** P < 0,05;**P < 0,01vs control*

The reduced glutathione (GSH) is known as one of the important cellular protectors. The decreased level of GSH is a sign of cytotoxicity due to the possible toxic metabolites formation. In the next experiments the effects of **7a-d** on GSH depletion were measured in isolated rat hepatocytes and compared to those of theophylline. Theophylline and **7d** decreased GSH level statistically significant by 62 %, compared to the control (non-treated hepatocytes); while the derivative**7b** decreased GSH level by 38 % (**Table 7**).

**Table 7. Effects of theophylline (100 µM) and 7a-d (100 µM) on GSH depletion in isolated rat hepatocytes**

| Group | GSH (nmol/$10^6$ cells) | Effect (%) vs control |
|---|---|---|
| Control | 21± 0,6 | 100 |
| Theophylline | 8 ± 1,7** | ↓ 62 |
| **7a** | 9±4,5* | ↓ 57 |
| **7b** | 13±3,5* | ↓ 38 |
| **7c** | 9±1,0 *** | ↓ 57 |
| **7d** | 8± 1,5 *** | ↓ 62 |

** P < 0,05;**P < 0,01;***P < 0,001vs control*

The resuts from the toxicity studies performed on isolated rat hepatocytes and microsomes showed that compound **7b** had the lowest toxic effects, while **7d** revealed higher toxicity among the newly synthesized 7,8-disubstituted theophylline derivatives. It is observed that the presence of two methoxy groups (**7b**) in the phenyl radical of the substitutes leads to significant decrease in the toxicity of **7b** in isolated rat hepatocytes. On the other hand, the introduction of methyl group (-$CH_3$) (**7d**) on α-position in the phenyl-ethyl-amino fragment leads to statistically

significant increase of the toxicity in isolated rat hepatocytes, with the most prominent effects on the LDH leakage – with 317 % and on GSH reduction – with 62 %.

## CONCLUSION

The synthesis of four new 7-benzyltheophylline8-thioglicolic acid amides was presented. The structure of the newly obtained compounds was proven by IR, $^{1}$H NMR and $^{13}$C NMR spectral analysis. The purity of the synthesized derivatives was elucidated by TLC characteristics and melting points. The cytotoxicity, antiproliferative activity and the *in vitro* effects on sub-cellular and cellular level of the synthesized compounds were evaluated. The results show, that compounds **7b** and **7d** perform highest effects, where **7b** displayed lowest toxicity in all evaluated parameters, while **7d** – highest. It was suggested, that the difference in the hepatotoxicity of **7a-d** and theophylline might be due to difference in their structure and metabolism, where the inclusion of an electrophile substituent resulted in decresment of the toxicity in isolated rat hepatocytes, compared to the one for the initial theophylline.

Within the series **7a** proved to be the most active antiproliferative agent, while **7b** and **7d** demonstrated only marginal activity in HL-60 and K-562 cell lines; **7c** was the least active structure in all cell lines.

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